

SCIENTIFIC OPINION

by **Prof. Maria Bogomilova Angelova-Dyankova**, DSc
on a dissertation submitted for the educational and scientific degree "doctor"
in the professional field: 4.3. Biological Sciences, doctoral program
Microbiology

Author: **Lena Rumenova Ilieva**

Topic: **Antimicrobial susceptibility and colonisation and invasiveness
factors in nasopharyngeal isolates of *Staphylococcus* spp.**

Scientific supervisor: **Prof. Dr. Sonya Kostadinova Trifonova**

1. General presentation of the procedure and the doctoral student

By order No. RD-22-1084 of 29.05.2026 of the Rector of Plovdiv University "Paisiy Hilendarski" (PU), I have been appointed as a member of the scientific jury to ensure a procedure for the defense of a dissertation on the topic "Drug sensitivity and factors of colonization and invasiveness in nasopharyngeal isolates *Staphylococcus* spp." for the acquisition of the educational and scientific degree "doctor" in the field of higher education 4. Natural sciences, mathematics and informatics, professional field 4.3. Biological sciences, doctoral program Microbiology. The author of the dissertation is Lena Rumenova Ilieva - a full-time doctoral student at the Department of Biochemistry and Microbiology, with scientific supervisor Prof. Dr. Sonya Kostadinova from PU. The set of materials presented by Lena Ilieva on an electronic medium is in accordance with Art. 36 (1) of the Academic Staff Development Regulations.

Lena Ilieva graduated from secondary school in 1996 at Aleko Konstantinov Secondary School in Svishtov. In 2001, she graduated with a Master's degree in Biology from the Plovdiv University, after which she successively worked as a biologist in a baby food production company, a microbiologist in a medical diagnostic laboratory, and an assistant at the Institute of Food Preservation and Quality in Plovdiv.

2. Relevance of the topic

Staphylococcus aureus is a species that is very often associated with increasing bacterial resistance to antibiotics. Due to its relatively high virulence and its great plasticity, it easily adapts to different environmental conditions. This species occupies a special place in the group of the most important bacteria (ESKAPE) causing infections. It has been proven that nasal carriage plays a key role in the pathogenesis of *S. aureus* infections. The pathogenic success is mainly due to virulence factors, which are most often active surface-located proteins and allow it to adhere to tissues, evade immune responses and damage host cells. In addition, its ability to form biofilms protects it from antimicrobial agents. All this forms a serious health problem that requires a comprehensive approach to the study and use of new biologically active compounds. In recent years, much evidence has been obtained about the diverse benefits of lactic acid bacteria (LAC) for human health. In this context, postbiotics are attracting increasing interest as natural antibacterial agents with the prospect of being included in new strategies to combat resistant pathogens and their ability to form biofilms.

It is this problem that formulates the scientific focus of Lena Ilieva's work, and this is an indication of its relevance and significance. The dissertation thesis is focused on obtaining new knowledge about the participation and pathobiological potential of *S. aureus* in symptomatic upper respiratory tract infections and finding new means to overcome antibiotic resistance.

3. Knowledge of the problem

The dissertation is constructed in a traditional form with the relevant sections. It is written on 207 standard computer pages, including 147 pages of text, 18 pages of literature and 42 pages of appendices. The literature review is prepared purposefully and specifically and takes into account all aspects of the study. It reflects 377 literary sources, which reflect the achievements on the problem to date. The text is characterized by a thorough study of the bibliographic sources and a creative approach to what is reflected in them. Over 45% of the cited literature is from the last 5-6 years, which gives the review a modern sound and emphasizes the relevance of the topic. The available literary data on the role of *S. aureus* in upper respiratory tract infections, the mechanisms of colonization, the formation of biofilms and the relationship with antibiotic resistance are discussed in detail. The approaches for molecular identification and genomic characterization of the *Staphylococcus aureus* species, as well as control strategies through decolonization, are presented. I would like to emphasize the importance of the subsections for new approaches in this direction and the importance of the ICD. At the end of the review, a subsection "Summary" is included, which is a very good idea and makes it easier for the reader to perceive the idea of the doctoral thesis more clearly.

The purpose of the dissertation corresponds to the relevance of the problem. It is clear and well formulated. To achieve this goal, 5 specific tasks have been formulated, which include all the mandatory stages of such a study.

4. Research methodology

The "Materials and Methods" section demonstrates a wide range of methods tailored to the specific requirements of the experiment and meets the focus of the dissertation thesis. Classical and modern, microbiological, molecular biological, biochemical and statistical methods were used. The development includes a huge number of nasal and throat samples from individuals with upper respiratory tract infections, and the isolated strains were identified by conventional and molecular genetic methods. I would like to highlight the experiments based on conventional PCR for gene amplification, full genome ONT sequencing, genome-based identification and multilocus sequence typing. The doctoral student has also applied confocal laser scanning microscopy and scanning electron microscopy, determination of probiotic potential with all its mandatory tests, purification of fractions with FPLC, electrophoretic separation, etc. All of them are sufficient grounds for reliability and precision.

5. Characteristics and evaluation of the dissertation work and contributions

The section "Results and discussion" includes extensive experimental material, which is characterized by logical consistency. The individual stages are developed in sufficient detail and are illustrated with 10 tables and 33 figures, most of which are complex.

The first stage of the development presents an in-depth study of the structure of nasopharyngeal infections for a period of 4 years based on 27,959 clinical samples, of which 6657 have proven microbiological growth and dynamics depending on epidemiological and clinical-organizational factors. The resulting model is similar to the global trend and takes into account the dominance of childhood, as well as the connection with seasonality. The etiological structure of this model clearly outlines the importance of *Staphylococcus aureus*, determined by correlation analysis of the relative shares of all isolated bacteria.

To identify the isolates of the genus *Staphylococcus*, the doctoral student applied a combined strategy of phenotypic and biochemical tests with sequencing of species-specific genes and complete genome sequencing. The obtained results create a serious scientific basis for the correct construction of the phylogenetic tree of clinical isolates belonging to the species *Staphylococcus aureus* and prove a well-expressed clonal population structure with multiple evolutionary lineages.

In response to the set goal of the dissertation, the doctoral student assesses the current situation regarding the resistance of local isolates of *Staphylococcus aureus* and the safety of the applied therapeutic regimens. A large-scale study of the drug resistance of 450 isolates to 17 antimicrobial agents selected according to EUCAST was conducted. Of interest are the different levels of resistance established depending on the type of antibiotics, which outline a specific antimicrobial profile of *Staphylococcus aureus* with significance for its local phenotypic characteristics, its ability to colonize and its role in the spread of resistant strains. A very important part of the dissertation is the *in silico* analysis of the resistome in *Staphylococcus aureus* using the ONT platform and the proven importance of the genetic potential for resistance. The doctoral student discusses the advantages of full genome sequencing compared to genotypic analysis, outlines the difference in the correspondence between genotype and phenotype and the dependence on expression, genetic background and accompanying mutational mechanisms. Furthermore, it provides compelling evidence of a correlation between resistance to a specific antibiotic and the presence of the responsible genes in 10 *S. aureus* strains included in the whole genome sequencing experiment. This is essentially new information about the importance of the resistome and the need for a joint phenotypic and genotypic study of the problem.

I would like to emphasize the importance of the following section, which adds a high-methodical genetic characterization of the virulence potential of nasopharyngeal isolates of the *S. aureus* species. The virulence potential, the importance of genes associated with pathogenesis, and the diversity of additional determinants in individual strains are outlined. A clearly expressed block of factors involved in the interaction between the pathogen and the host and those that help evade local immune control has been established. The experimental data have been verified by *in silico* analysis based on a specially developed PCR panel in a wide range of isolates. The results on the capacity of clinical isolates to form biofilms, as a major factor for their resistance to antibiotics, are reflected in a logical sequence. The genetic basis and dependence on environmental conditions, as well as the relationship between the potential for adaptation and the potential for persistence, have been proven.

The following sections (6, 7 and 8) are dedicated to the current task of finding new agents with anti-staphylococcal activity. On more than 45 pages, the doctoral student presents a detailed characterization of the antagonistic potential of selected MKB strains against nasopharyngeal isolates of *S. aureus* and the mechanisms of action through a combined approach, including phenotypic screening, genomic characterization and analysis of postbiotic products. After a broad screening of the antibacterial activity of 12 MKB strains, their capacity to inhibit the development of 60 *S. aureus* isolates was assessed. The conducted full genome analysis of selected MKB strains revealed a diverse functional repertoire of genes. Furthermore, in silico analysis of the genome structure outlines a stable core of genes associated with probiotic potential and at the same time the presence of genetic elements determining different strategies of antibacterial action. The presence of well-organized clusters for the synthesis of bacteriocins and post-translationally modified peptides relevant to the antimicrobial strategy has been proven genetically determined. Based on the results obtained, the doctoral student selected three strains with high probiotic potential and further proved it through the mandatory tests for such activity.

In accordance with the most current research directions, the doctoral student is investigating the possibility of applying postbiotics (cell-free supernatants, BS) from selected MKB as anti-staphylococcal agents. The presence of fatty acids, the potential for limiting biofilm formation and suppressing planktonic growth has been experimentally established. An important part of the development are the experiments to prove the protein nature of the antibacterial activity by fractionating BS from *L. plantarum* IZITR_24 and *L. paraplantarum* IZITR_13, their concentration and enrichment. Of interest are the data on the role of the low-molecular protein fraction and the presence of bacteriocin-like components in inhibiting the development of *S. aureus*. The mechanism of action of the lyophilized cell-free supernatants has also been proven at the microscopic level.

The presentation of the results is combined with a skillful discussion of all stages of the development based on appropriate literature sources. It is worth noting the extensive analysis of the results obtained and the correctness in formulating the conclusions from the individual experiments. The doctoral student clearly distinguishes between direct evidence and the need for additional research. For a reliable assessment of the data, she coherently uses different, complementary approaches at a modern methodological level. The discussion also demonstrates Lena Ilieva's in-depth knowledge in this area.

At the end of the dissertation, there is a separate section "Conclusion", which summarizes the correctly obtained data and emphasizes the contributions of the doctoral student. This makes it easier for the reader to orient himself very accurately in the work and to perceive the achievements in it.

I would like to emphasize with pleasure the very good layout of the dissertation, the concise scientific style in which it is written, the correct reflection of the results in tables and figures, as well as their professional presentation.

In my opinion, the conclusions are a logical consequence of the experimental data and provide the necessary information about the value of the conducted research. I also accept the formulation of the contributions and I want to emphasize their importance in a theoretical and

scientifically applied aspect. In essence, they relate to obtaining new information through modern methods regarding the drug sensitivity and pathobiological characteristics of nasopharyngeal staphylococcal isolates and characterization of new effectively acting natural compounds with antibacterial activity based on postbiotics from the ICD.

Everything said so far emphasizes once again the significance and relevance of the dissertation.

6. Evaluation of the publications on the dissertation and the personal contribution of the doctoral student

The data from the dissertation have been included in 2 scientific articles, published in renowned specialized journals: Microorganisms with IF 4.7 and quartile Q1 and International Journal of Molecular Sciences with IF 5.6 and quartile Q1. This gives me reason to believe that Lena Ilieva's results have become known to our and the international scientific community. She is the first author in 1 scientific publications, which suggests her significant share in the development of the dissertation work.

7. Critical remarks, recommendations and questions

I have no serious critical remarks, the work as a topic and execution deserves admiration. I have the following questions for the doctoral student:

1. How do you plan to disseminate the developed complex model for the combined application of modern approaches in the characterization of clinical isolates of *Staphylococcus aureus* in microbiological practice?
2. In your opinion, is it mandatory for postbiotics/lyophilized cell-free supernatants from MKB, proposed as agents with antibacterial activity, to be obtained from proven probiotic strains?
3. In what direction should research continue on the application of postbiotics for modifying the growth and formation of biofilms by *Staphylococcus aureus*?

CONCLUSION

In conclusion, I would like to emphasize that the doctoral student has fulfilled the requirements of the Law on the Scientific and Technological Development of the Republic of Bulgaria, as well as those in the Regulations to it of the University "Paisii Hilendarsky" for acquiring the educational and scientific degree of "doctor". The material presented by Lena Ilieva is dissertable, the topic is topical and offers a modern level of an important issue for theory and practice. The experiments conducted were methodically set up correctly, the results obtained are reliable and are a solid basis for further scientific and applied developments. A huge amount of experimental work has been carried out, the problem posed has been studied in many ways and in detail at a modern level, significant scientific contributions have been made, including those with serious applied significance. To this characteristic of the dissertation work, I would like to add that, in my opinion, Lena Ilieva leaves the doctoral program as a well-prepared specialist in the field of microbiology, has mastered a large number of modern methods, has gained experience in interpreting scientific data.

Due to the above, I confidently give my positive assessment of the conducted research, presented by the above-reviewed dissertation, abstract, achieved results and contributions and propose to the esteemed scientific jury to award the educational and scientific degree "**DOCTOR**" to **LENA RUMENOVA ILIEVA** in the field of higher education: 4. Natural Sciences, Mathematics and Informatics; professional field 4.3. Biological Sciences, doctoral program Microbiology.

07/26/2026

Sofia

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/prof. Maria Dyankova, DSc/